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EVALUATION OF NEW ANTIMALARIAL DRUG COMBINATIONS

by:
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A thesis submitted to the faculty of The University of Mississippi,
In partial fulfillment of the requirements of
The Sally McDonnell Barksdale Honors College.

University, MS
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ABSTRACT

Malaria is a disease that has affected people throughout the world for centuries and is still responsible for over 200 million clinical cases per year. While a number of developed countries have eradicated the disease, its prevalence in tropical regions poses a significant global health challenge. The disease is caused by the *Plasmodium* genus, an obligate, intracellular parasite that is carried by the female *Anopheles* mosquito. While there are effective antimalarial drugs in clinical use, *Plasmodium*'s resilient nature has allowed it to develop resistance to multiple drugs. This fact makes it imperative to seek out novel drugs to combat resistant strains. Combination therapies are one effective way to treat malaria and prevent the incidence of drug resistance. The most commonly used class of combination therapy is the Artemisinin Based Combination Therapy. However, there is evidence that artemisinin resistance could be occurring in the field.

In this study, new combinations were tested between the standard antimalarial drugs namely, chloroquine (CQ) and artemisinin (ART) along with the experimental antimalarial drugs namely an artemisinin dimer oxime (DIOX) and NPC1161B (NPC) (an 8-aminoquinoline). The combinations were tested *in vitro* using the SYBR Green-I based fluorescence assay with a drug-resistant W2 strain of *Plasmodium falciparum* (*P.f.*) in a checkerboard assay template. Resistance of Pf (W2) towards CQ was confirmed. Both DIOX and NPC showed partial antagonistic effect on the antimalarial action of CQ and artemisinin. Further *in vivo* evaluation of these combinations in rodent malarial models would be important for confirmation of these results.

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LIST OF ABBREVIATIONS

CDC	Center for Disease Control
WHO	World Health Organization
<i>P.f.</i>	<i>Plasmodium falciparum</i>
RBC	Red blood cell
CQ	Chloroquine
ART	Artemisinin
DIOX	Dimer Oxime
NPC	Natural Products Center 1161B
LDH	Lactate Dehydrogenase

Chapter I: Background and Significance

1.1 Introduction

Malaria is a disease that has affected the people of the world for thousands of years. According to the Center for Disease Control (CDC), 3.2 billion people live in areas that are at risk of transmission, and in 2016, estimated 216 million clinical cases of malaria were reported (WHO, 2017). This incidence of infection resulted in nearly 445,000 fatalities. Of the infected population, the most vulnerable are young children, pregnant women and travelers from non-endemic regions (Impact of Malaria, 2017). As shown in the global malaria distribution map (*Figure 1*), infections are prominent in tropical regions, especially in India and Sub-Saharan Africa.

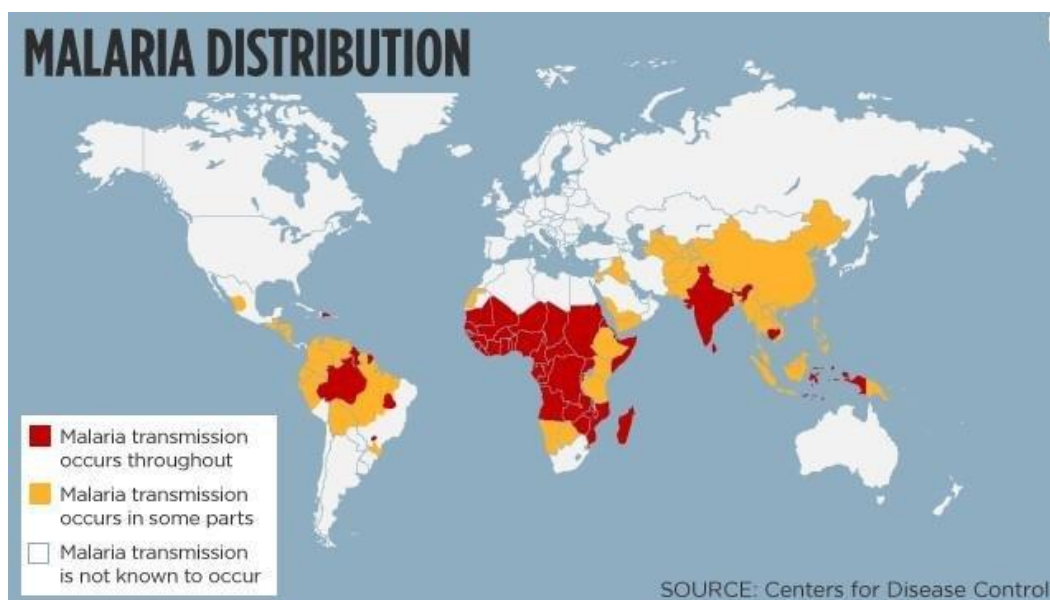


Figure 1: CDC map representing the global distribution of human Malaria cases from the Center for Disease control

Limited numbers of drugs and no viable vaccines to treat or prevent the disease make malaria a prominent global health challenge. Additionally, increasing resistance of

the malaria parasite to commonly used drugs creates a sense of extreme urgency to discover and incorporate new antimalarial drugs that are both cost-effective and easily administered

1.2 History

Malaria has been with humans throughout history, but it was not until the many technological advances of the past century that medicine's understanding of the origin, transmission and life cycle of the disease could be understood. Although many people contracted malaria and were familiar with its symptoms, the true cause of the disease was not discovered until Alphonse Laveran, Ronald Ross, Battista Grassi, and their colleagues identified the malaria parasite in the 1880's and 1890's (Meshnick and Dobson, 2001). Additionally, Ross was awarded the Nobel Prize in 1902 for demonstrating that the malaria parasite is transmitted to humans through a mosquito vector (The History of Malaria 2017). This discovery allowed scientists to better understand how to combat the symptoms and spread of malaria.

Like many early drugs, the first antimalarial drugs, namely quinine and artemisinin, were discovered hundreds of years before the disease was understood on a physiological and biochemical level. The earliest remedies for malaria came from the bark of the cinchona tree and the qinghao plant; these remedies were later synthetically separated as quinine and artemisinin respectively (Meshnick and Dobson 2001). During the conquest of the new world and the spread of imperialism by Europeans, the interest for developing effective antimalarial drugs reached new heights. However, it was not until the twentieth century that resistance to malaria drugs became evident. This had proved to be a major challenge that has continued to this day creating a great need for new antimalarial drugs.

1.3 The Parasite

Members of the *Plasmodium* genus are responsible for the symptoms of malaria. *Plasmodium* parasites are obligate intracellular protozoan parasites that have a complex life cycle. This includes time spent in both humans and female the *Anopheles* mosquitos, malaria's primary vector of transmission. There are four species of *Plasmodium* that cause the disease in humans: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* (WHO 2017). *P. falciparum*, which is the species used in the experiments presented in this thesis, by far causes the most clinical cases of malaria worldwide resulting in over 200 million clinical cases of malaria per year (Lui et al., 2016). Because *P. falciparum* is the most virulent species it naturally draws the most attention for research, but the other species are just as dangerous. *P. vivax* is estimated to cause anywhere from 35 million to 80 million cases of malaria per year, and these infections, like *P. falciparum*, are capable of causing morbid disease states that can easily result in fatality (Galinski 2008). Although *P. ovale* and *P. malariae* can independently infect humans, these species most commonly infect opportunistically along with *P. falciparum*, and additionally, it is much more difficult to detect these species by light microscopy techniques (Rutledge et al 2017, Doctor et al. 2016). Because of these facts, there is less research and discovery focused on these species because of the pressing need to discover new drugs to combat drug resistant strains of *P. falciparum*.

1.4 Life Cycle

The *Plasmodium* parasites have a complicated life cycle that includes a period in both humans and female *Anopheles* mosquitos. The parasites also have both an asexual and sexual stage of growth and reproduction throughout their life cycle. *Figure 2* provides an overview of this complicated life cycle.

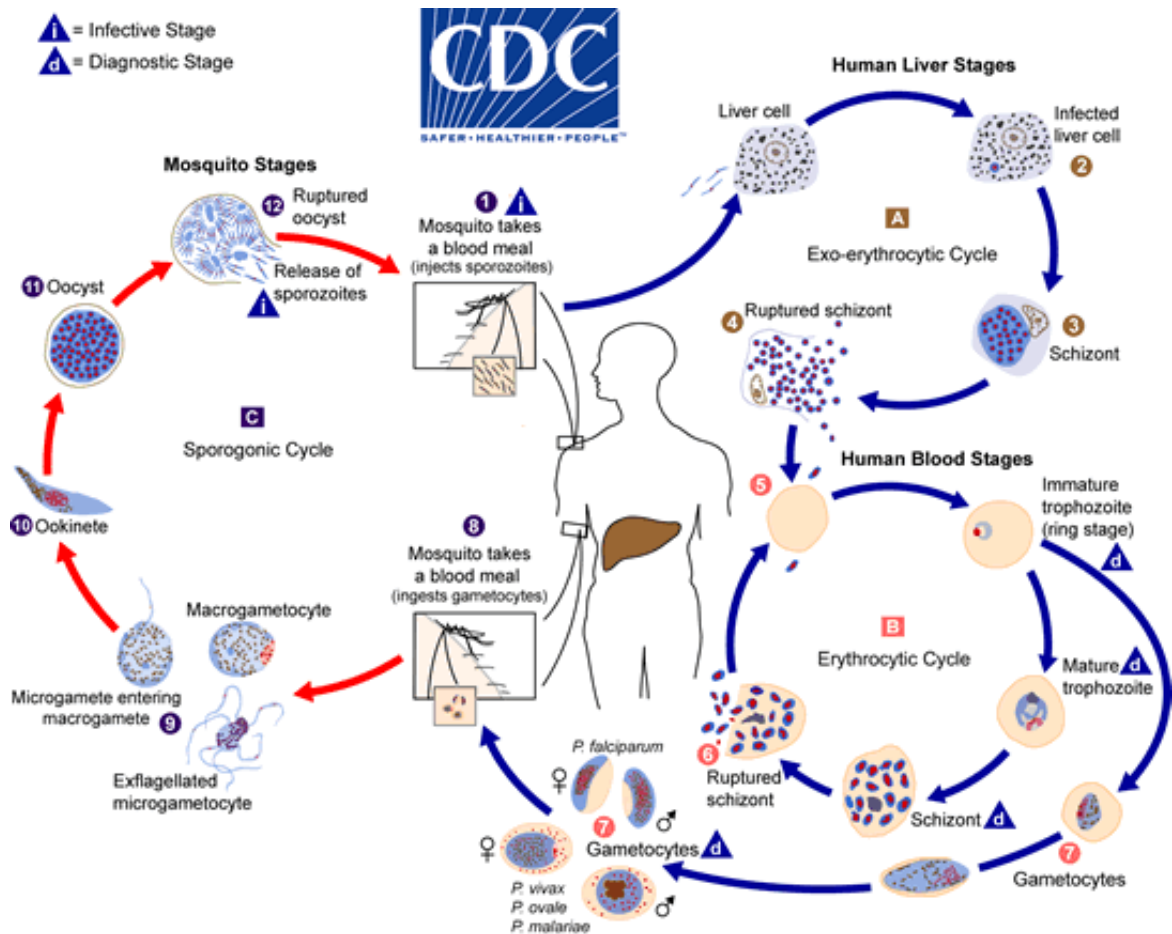


Figure 2: Life cycle of Plasmodium from the Center for Disease Control

There are three primary stages in the parasite's life cycle: The Exo-Erythrocytic Cycle, the Erythrocytic Cycle, and the sexual Sporogonic Cycle. The life cycle as depicted in *Figure 2* begins in the asexual stage when an infected female *Anopheles* mosquito bites

a human and introduces sporozoites into circulation. After arriving in the bloodstream, the parasites circulate and are taken up by the liver. Within the liver hepatocytes, the parasites enter the schizont stage of development where they multiply rapidly eventually causing cell rupture and release of thousands of infectious merozoites into the bloodstream. At this stage, *P. vivax* has the unique ability to remain dormant and replicates in the liver allowing for subsequent infection, weeks to even months after initial exposure to the parasite (Siciliano, 2015). These merozoites then infect human erythrocytes and subsequently progress to the ring stage, and at this point have two available pathways.

The first pathway involves maturation into a mature trophozoite, which then again progresses into many infectious merozoites that continue the erythrocyte infection stage. During the Erythrocytic Cycle, the parasite is dependent on human Red Blood Cells (RBCs) because it feeds on the Oxygen carrying protein hemoglobin (Miller, 2013). In *P. falciparum* and *vivax*, this cycle is completed in 43-48 hour intervals causing the observed 2 day spike in fever and other symptoms associated with the clinical malaria infections (Fujioka 1999).

The second available pathway is the progression to the sexual gametocyte stage of the life cycle. A mosquito that is feeding on an infected human can then take up the gametocytes. Within the mosquito, the parasite goes through its process of sexual exchange of genetic material and multiplies into many sporozoites preparing the parasite for infection during the mosquito's next blood meal. The different stages of the life cycle can be easily identified by light microscopy and are presented in *Figure 5* in chapter II.

1.5 Clinical Presentation

While some complications can occur during the liver stage of the infection, the most prominent symptoms of malaria occur during the Erythrocytic Cycle. Major symptoms of the disease are high fever, sweating, and moderate to severe chills; while headache, diarrhea and vomiting may also be present in some cases. These symptoms do not usually directly cause fatalities, but they can lead to more serious complications that present in severe cases of the infection. Severe complications include: cerebral malaria causing swelling of the brain, breathing problems caused by pulmonary edema, liver or kidney failure, low blood sugar, and severe anemia resulting from the destruction of erythrocytes during the blood stage of infection (Mayo Clinic, 2018). These severe complications are very difficult to combat in the poor, rural areas of the tropics and Africa and are responsible for malaria's extremely high mortality rates.

Because the malaria life cycle and infection is multifaceted and complicated, a variety of treatment methods are required to overcome the disease. Management of symptoms in severe cases is imperative to survival and can be accomplished through: intravenous fluids to combat dehydration due to vomiting and diarrhea, blood transfusion to reverse anemia caused by the Erythrocytic Cycle, and dextrose is provided intravenously to reverse the effects of low blood sugar (White et al., 2014). There is also a variety of antimalarial drugs available that can effectively clear the body of infection. However, these drugs are sometimes inaccessible to the populations in need and the parasite can become resistant to certain drug therapies. The discussion of antimalarial drugs is presented in the following section. Prevention of the disease is highly recommended and can be accomplished through a variety of methods. A popular approach to prevention is the

“A.B.C.D.” method: Awareness of risk, Bite avoidance, Compliance with prophylaxis, and Diagnosis after fever (Genton, 2012). Awareness education programs help populations to avoid bites through the use of bed nets, insect repellent and management of standing water sources, which can support mosquito reproduction (Burke 2017). These measures can help limit the transmission of the disease, however malaria will nevertheless still be a major problem in endemic areas and it is therefore imperative to continue to develop new drugs in order to combat the deadly disease.

1.6 *Malaria Drug Discovery*

Because *Plasmodium* has the potential to adapt and become resistant to antimalarial drugs, it is imperative to develop and utilize methods to discover novel drugs in order to fight the ever-adapting genome of the parasite. These methods include screening for new drug candidates, *in vitro* and *in vivo* models to test the efficacy of drugs, and diagnostic methods to determine the amount of parasitemia, or relative number of infected RBCs, in a model.

1.6.1 *Antimalarial Drugs*

The first synthetic antimalarial drug discovered was chloroquine, which was discovered in 1934. Chloroquine is considered one of the most important drugs in the history of infectious disease treatment, and along with the development of DDT, there were hopes that the disease could be potentially eradicated from the world (Wellems, 2001). While this feat would be monumental, it was determined to only be hope. Like all living things, *Plasmodium* is a resilient parasite and eventually mutated, developing resistance to this widely distributed antimalarial. *P. falciparum* chloriquine resistance was first recognized in the 1950's and has nearly spread across the globe. Additionally, resistance

in *P. vivax* was discovered in 1989 and has since spread as well (Baird, 2018). The effects of chloroquine resistance were noticeably drastic. Hospital studies conducted in Africa in the 1980's showed a 2-3 times increase, and 6 times in the case of Senegal, increase in malaria deaths that could be attributed to the parasite's development of chloroquine resistance (Trape, 2001). The development of chloroquine resistance caused the pharmaceutical community to eagerly begin the search for new drugs to combat the disease.

Artemisinin was one of the drugs that was discovered in response to the chloroquine resistance crisis that occurred in the mid twentieth century. It was isolated in the 1960's by Chinese scientists and in 2006 there was a worldwide demand for nearly 100 million artemisinin-based combination therapies (Covello, 2008). The World Health Organization switched to this new combination treatment strategy in 2005 and it has proved to be effective in saving many lives (Tu, 2011). These treatments are currently regarded as the most effective treatments for malaria and are being utilized across the world in malaria endemic regions.

Artemisinin's primary mechanism of action takes advantage of the parasite's reliance on erythrocyte hemoglobin for nutrients. Normally, the drug has limiting effects on humans because it is activated by the heme, which is typically locked away inside the hemoglobin of RBCs. However, during the multiplication of malaria parasites within the host RBCs, hemoglobin is rapidly digested and free heme is present in infected RBCs; this allows artemisinin to become active and enact cytotoxic effects on the parasites (Wang, 2017). While there have yet to be confirmed cases of artemisinin insensitive strains of malaria parasites, there have been observations in the last decade that in some cases of malaria are beginning to take longer than normal to clear the parasite with artemisinin-

combination therapies (Sibley, 2015). This trend suggests that, like chloroquine, malaria parasites have the potential to develop resistance to artemisinin and artemisinin-combination therapy treatments in the coming years.

Global Portfolio of Antimalarial Medicines

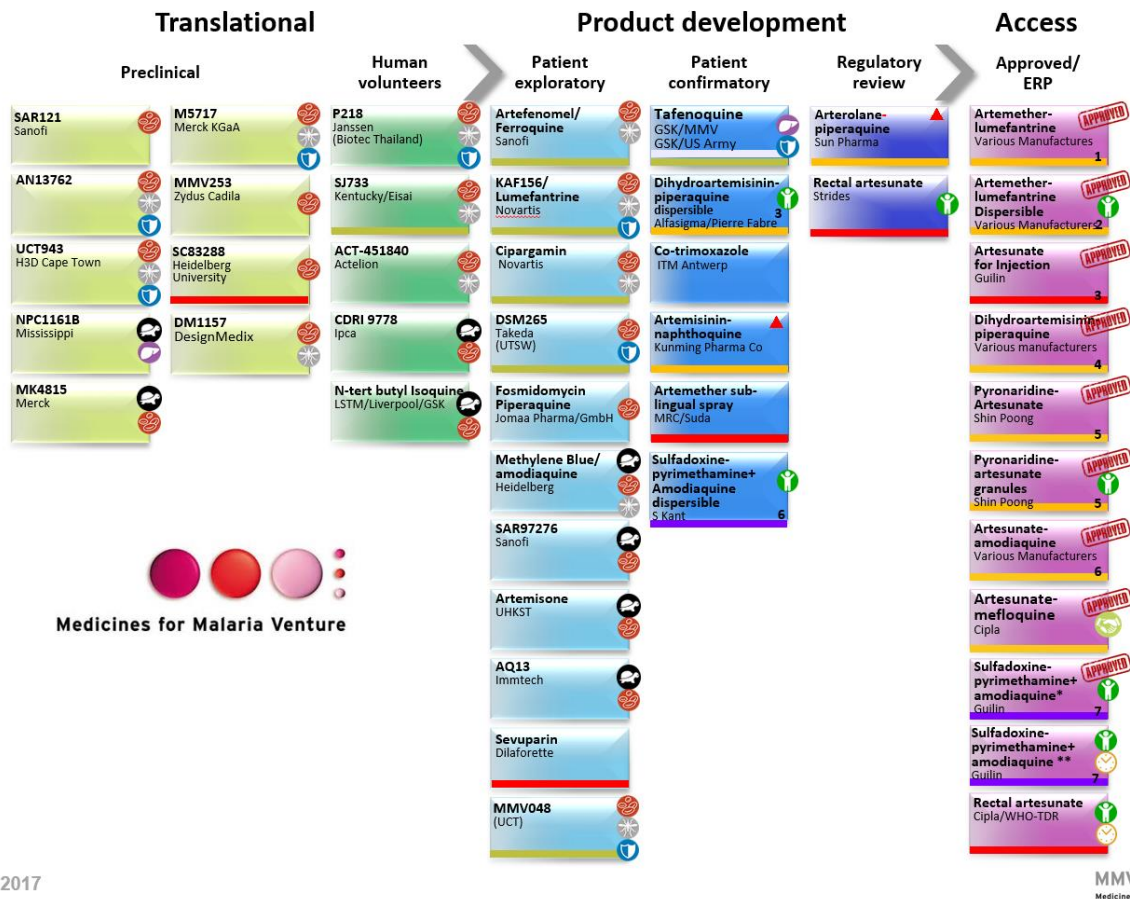


Figure 3: A portfolio of the current antimalarial drugs in each stage of development available from Medicines for Malaria Venture

The Medicines for Malaria Venture (MMV) was established as a Public-Private-Partnership for antimalarial drug development. MMV has established a network of more than 160 partners world-wide, which include the research labs from the academic institutions, global health agencies, government organizations and pharmaceutical companies. With these partnerships, MMV has established one of the largest antimalarial drug discovery portfolios shown in Figure 3. This portfolio includes the candidate's drugs in clinical development especially for medical needs in malaria. This includes the medicines for children and pregnant women.

1.6.2 Antimalarial Drug Discovery Models

The experimental models are important tools for pharmacological drug discovery. They provide the necessary tools to test the efficacy and safety of novel drug targets in a more controlled and ethical environment. Researches searching for new antimalarial drug therapies have both an *in vitro* and *in vivo* option for testing the effectiveness of new compounds and drug combinations.

1.6.2.1 *In vitro* Malaria Model

One of the greatest obstacles that early researchers faced was the inability to effectively culture the *Plasmodium* parasite. This forced early research to be primarily clinical and made it difficult and potentially unethical to attempt to use new antimalarial drugs on human subjects. Currently, the practice of culturing *P. falciparum* in the laboratory is common and is extremely effective for screening potential new drug targets. The common protocols to culture *P. falciparum* use A+ human erythrocytes, specific cell culture media, and A+ human serum, which can be replaced with a lipid- rich serum albumin fraction. The *P. falciparum* cultures are maintained in a controlled incubator environment of 5% CO₂, 5% O₂, and 90% N₂, at a temperature of 37° C (Singh et al., 2007).

There are a variety of culture strains that can be chosen to be grown in the laboratory. The strains used in this experiment are the D6 and W2 strains. The D6 *P. falciparum* strain originates from Sierra Leone and is effectively utilized as a baseline because it has no known drug resistance (Fidock, 2004). The W2 *P. falciparum* strain has its origins in Indochina and is resistant to Chloriquine (CQ), Quinine (QN), Pyrimethamine (PYR), Sulfadoxine (SDX), and Cycloguanil (CYC), making it an important strain in

showing the effectiveness of a new drug in comparison to a drug that has evolved resistance (Fidock, 2004).

Once a sufficient amount of culture has been grown, the culture can be combined with varying drugs and concentrations in a 96-well plate in order to test the antiparasitic activity of the drugs in question (Sahu et al., 2014). The results of these assays can be measured by a variety of methods. Some of the major methods of analysis are: Light microscopy with Geimsa staining, *Plasmodium* LDH assay, SYBR green assay, and analysis by flow cytometry. These methods all measure the percent growth of the parasite with reference to the control wells and can be analyzed to obtain IC-50 and IC-90 values for the drugs used in the experiment. Some advantages of this model are: the ability to screen many compounds without the use of animals or humans, the ability to better control concentrations and utilize drug combination studies, better discernment for effects like addition, antagonism and synergism, and it is less expensive than other models. However, the physiological effects of a drug cannot be observed in an *in vitro* study, and having a nonliving model allows a drug that could be potentially toxic to animals to eliminate the parasite creating a type of false positive test result.

1.6.2.2 *In vivo* Rodent Malaria Model

Animal models are a vital intermediate between *in vitro* drug discovery assays and human clinical trials. Unlike *in vitro* trials, animal models allow researchers to observe both the anti-parasitic, as well as the biological and physiological effects of a drug. These models also provide a more accurate picture of the interaction of the drug with the real disease because it is able to progress through its natural host life-cycle and the host-immune defenses are present to assist with parasite clearance. The most common *in vivo*

antimalarial model is the mouse model. This is mainly due the availability of laboratory mice, the minimal dosing of drug required and relatively low cost compared to other screening models. There are currently four *Plasmodium* species that can be utilized in rodent models: *P. berghei*, *P. yoelli*, *P. chabaudi*, and *P. vinckei* (Fidock, 2004).

1.6.2.3 Combination Screening Assays

Pursuing the discovery of new antimalarial combination therapies is an important part in the worldwide battle against malaria. Combination therapies are able to increase efficacy, decrease time of treatment required, and decrease risk of resistance arising in the parasite (Kakkilaya, 2015). The most common form of combination therapies is the artemisinin-based combination therapies (ACT's). These therapies showed good results in the field, however a few incidences of resistance are being reported leading researchers to seek out new combination therapies for the future.

The discovery of new combinations is similar in practice to the screening methods used in discovering isolated drug therapies, but instead of testing one drug at various concentrations, drugs are tested in combination with each other in various concentrations in a “checkerboard” format as discussed more thoroughly in the *Materials and Methods* section.

1.7 Objective and hypothesis

Artemisinin combination therapies (ACTs) form the first line of treatment for complicated as well noncomplicated malaria. Recent studies have shown that efficacy of artemisinin may be improved with formation of artemisinin dimers. Artemisinin dimer oxime (DIOX) (Figure 4) was recently identified as a promising new antimalarial drug lead (ElSohly et al., 2018). In this experimental antimalarial drug the artemisinin molecules are linked by a polyethylene glycol spacer. The DIOX shown good activity against CQ-susceptible and CQ resistant strains of *P. falciparum*, lower IC₅₀ values *in vitro* when compared to artemisinin and also cured the disease in *Plasmodium berghei*- mouse malaria model. ACTs are not active against the liver stage malaria and also not effective against the malaria gametocytes, which are responsible for initiation of infection and transmission malaria respectively. NPC1161B, an 8-aminoquinoline (analog of primaquine) antimalarial (Figure 4) is currently under preclinical development. NPC1161B is a single R (-) enantiomer developed by the researchers at the NCNPR. NPC1161B is currently under late-stage preclinical development. In preclinical studies in animal models NPC1161B has shown better therapeutic profile compared to primaquine (which in clinical use) and also tafenoquine (which is under clinical development)

Hypothesis- The new experimental drugs act in combination with the established clinical antimalarial drugs and show better antimalarial efficacy. These new combinations are also active against drug-resistant isolates of the malaria. DIOX and NPC1161B (the experimental antimalarial drugs) were tested in combination with chloroquine and artemisinin (the most prominently used antimalarial drugs used in clinics).

Chapter II Materials and Methods

2.1 Test drugs and compounds

Chloroquine (CQ) and artemisinin (ART) were used as the standard antimalarial drugs in this study (Figure 4). NPC1161B, an 8-aminoquinoline analog developed by the researchers at the NCNPR, which is currently under late stage preclinical development (Tekwani and Walker, 2006) was tested as an experimental drug. Recent studies at the NCNPR have also identified some new orally active artemisinin dimer antimalarials (Elsohly et al., 2017). The artemisinin dimer oxime (DIOX) from this series was tested as another experimental antimalarial drug.

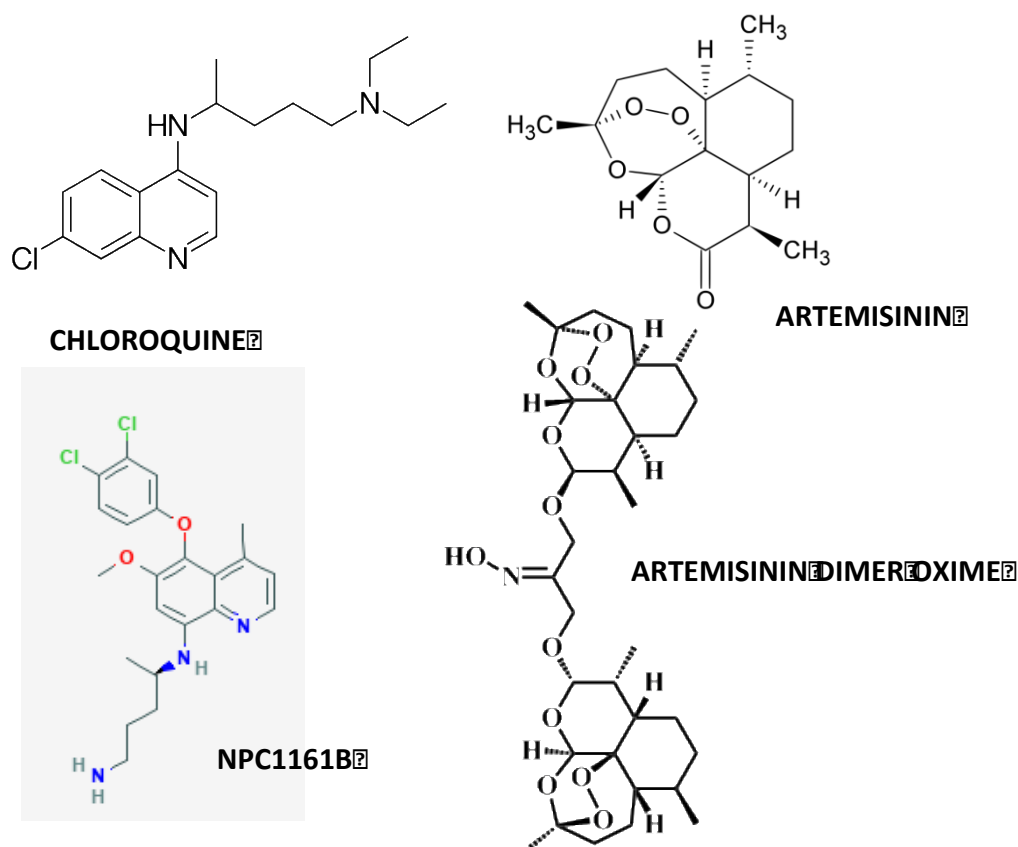


Figure 4: Standard and experimental antimalarial drugs tested as new antimalarial drug combinations.

2.2 Cell Culture

The *Plasmodium falciparum* laboratory strains of CQ-sensitive D-6 and -resistant W-2 were obtained from the Malaria Research and Reference Reagent Resource Center (MR4 now BEI Resources- www.beiresources.org) in the United States. *P. falciparum* (strain D6) was originally collected in the Sierra Leone and is generally considered drug-sensitive. *P. falciparum* (strain W2) was cloned from the Indochina III/CDC isolate originally derived from a Laotian patient who failed chloroquine therapy. The strain W2 is reported to be resistant to chloroquine and susceptible to mefloquine. These *P. falciparum* strains were grown in a culture of A+ human red blood cells obtained from the Mississippi Blood Services. The culture was maintained at a level of 5% hematocrit in complete malaria culture medium (RPMI 1640 with 60 µg/mL of amikacin, 2.2 g of sodium bicarbonate per liter of medium, 25 mM HPES buffer). The medium was replaced every 48 hours and the culture flasks were kept in an incubator environment of 37° C and maintained gaseous environment of 90% N₂, 5% CO₂, and 5% O₂.

2.3 Microscope Parasitemia Analysis

Throughout the process of maintenance the level of parasitemia exhibited by the *P. falciparum* cultures was monitored by light microscopy. When the medium was changed, blood smears from both the cultures of D-6 and W-2 strains were prepared for analysis. The slides were fixed in pure methanol for 30 seconds before being stained with Giemsa stain for 50 minutes. After the staining was complete, the slides were washed with distilled water and allowed to air dry. Sample images from these slides are presented below in *Figure 5*.

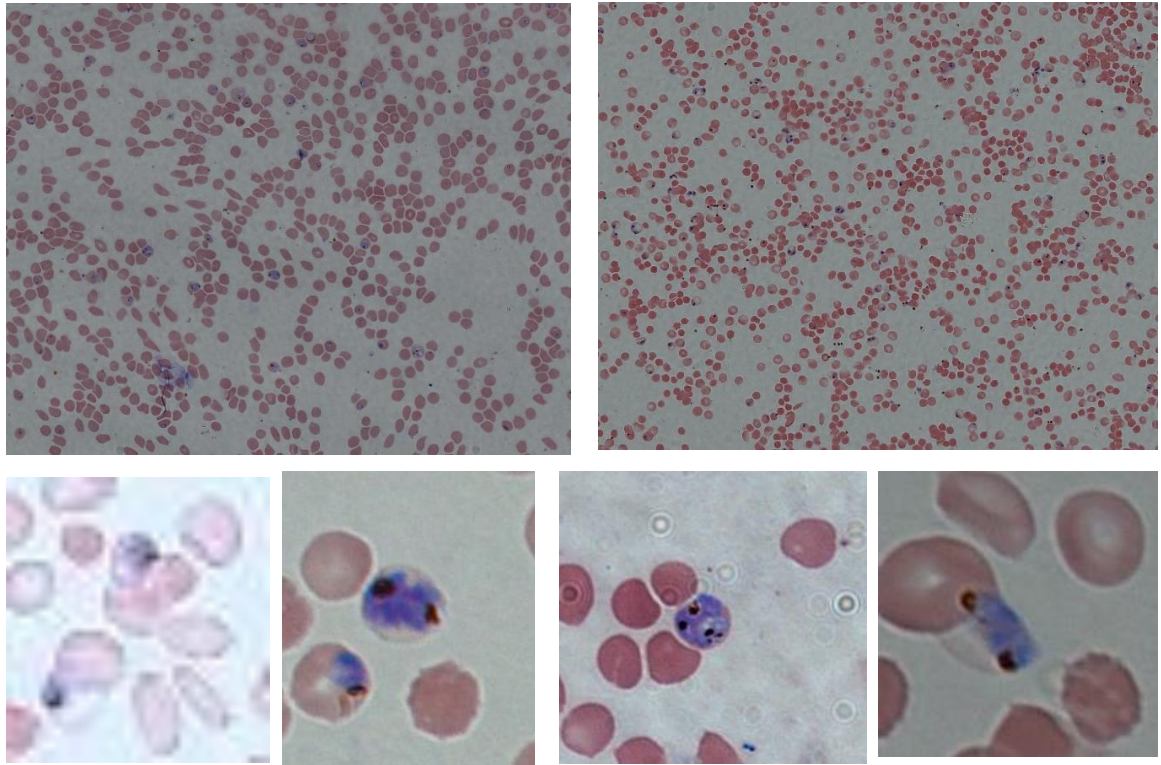


Figure 5: Sample slides of Geimsa stained P. falciparum culture (Top). From left to right: ring stage, trophozoite, schizont and gametocyte (Bottom). The images taken from the Geimsa stained smears of the P. falciparum cultures maintained at our lab.

The parasitemia analysis was conducted using NIS-Elements software to collect pictures from a Nikon Eclipse 90i light microscope equipped with Nikon Digital Sight cameras. ImageJ software was then used to count the total number and infected number of RBC's in order to calculate parasitemia. The total RBC count was taken by selecting; "binary", "fill holes", and "watershed", and then selecting "analyze particles" (Circularity 0.25-1.00, Pixels 500-infinity, display outlines). The infected RBC's in the ring stage only were then counted and divided by the total number of RBC's and multiplied by 100 to calculate the culture parasitemia. Before beginning the SYBR Green antimalarial assay,

the parasitemia was also counted to calculate the necessary dilution to use to achieve 2% parasitemia in 2% hematocrit for the SYBR Green assay.

2.4 *Antimalarial SYBR Green I-based fluorescence assay*

The antimalarial SYBR Green I-based fluorescence assay was used to determine the parasite's response to both the single drug treatment experiments and the combination study of drugs (Johnson *et al.*, 2007). This technique utilizes a fluorescent staining technique and a plate reader to determine the percent growth of the parasite in response to various drug treatments.

For the single treatment study, the ring stages of the D6 and W2 parasites were counted and the parasitemia was adjusted to 2% in 2% hematocrit. Stock solution of the drugs and test compounds were prepared in DMSO or deionized water. Each test compound was serially diluted in serum-free RPMI medium at eight experimental concentrations. The maximum concentrations for the CQ, ART, DIOX, and NPC were 500 nM, 250 nM, 100 nM, and 5000 nM respectively. The template for this test is presented in *Table 1* below. After initial setup, the treatment plates were incubated at the culture's standard conditions of 37° C, with 90% N₂, 5% CO₂, and 5% O₂ for 72 hours. Each well was then treated with SYBR Green and an RBC lysis buffer before being covered with the aluminum foil for complete darkness and placed on a shaker for 1 hour at room temperature. The fluorescence of each well was analyzed by the Fluostar Galaxy microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm at 20% gain. The gain adjustment determines sensitivity of the fluorescence measurement, which was standardized in the initial assays.

For the combination assays, the culture was maintained and prepared in identical manner to the single treatment experiment. However, instead of performing a serial dilution for each drug, a checkerboard assay was set up to test the percent growth of the *P. falciparum* in response to two drugs at varying concentrations. The following four combinations were tested: Chloroquine (CQ) with Dimer Oxime (DIOX), Artemisinin (ART) with DIOX, CQ with NPC1161B (NPC), and ART with NPC1161B. The maximum concentration for CQ, DIOX, and ART was 200 nM, and the maximum concentration used for NPC1161B was 5,000 nM. An example template for the combination plate of CQ and DIOX is presented in *Table 2* below. After the initial setup of the plates, the combination assay was performed in the same manner as the single treatment assay described above. The percent growth was also analyzed using Xlfit© and the data was represented as IC-50 values.

Table 2: This table is the template for the combination assay of CQ (in blue) and DOX (in black).

Medium	RBCs	P.f.	DMSO	3.12+ 3.125	6.325 + 3.125	12.5 + 3.125	25.0 + 3.125	50.0 + 3.125	100.0 + 3.125	200.0+ 3.125	3.125
Medium	RBCs	P.f.	DMSO	3.125 + 6.25	6.325 + 6.25	12.5 + 6.25	25.0 + 6.25	50.0 + 6.25	100.0 + 6.25	200.0 + 6.25	6.25
Medium	RBCs	P.f.	DMSO	3.125 + 12.5	6.325 + 12.5	12.5 + 12.5	25.0 + 12.5	50.0 + 12.5	100.0 + 12.5	200.0+ 12.5	12.5
Medium	RBCs	P.f.	DMSO	3.125 + 25	6.325 + 25	12.5 + 25	25.0 + 25	50.0 + 25	100.0 + 25	200.0 + 25	25.0
Medium	RBCs	P.f.	DMSO	3.125 + 50	6.325 + 50	12.5 + 50	25.0 + 50	50.0 + 50	100.0 + 50	200.0 + 50	50.0
Medium	RBCs	P.f.	DMSO	3.125 + 100	6.325 + 100	12.5 + 100	25.0 + 100	50.0 + 100	100.0+ 100	200.0+ 100	100.0
Medium	RBCs	P.f.	DMSO	3.125+ 200	6.325+ 200	12.5 + 200	25.0 + 200	50.0 + 200	100.0+ 200	200.0+ 200	200.0
Medium	RBCs	P.f.	DMSO	3.125	6.25	12.5	25.0	50.0	100.0	200.0	Medium

2.5 *Analysis of Data*

The raw data from the plate reader was collected and the percent growth was calculated by comparing the fluorescence values of the treatment wells to the value of the control wells. The percent growth and corresponding concentration were paired and graphed using Xlfit© software on Excel. This calculation provided IC-50 values with standard deviation and presented the data in a growth response curve. P values were calculated from the IC-50 values of the combination treatment using the following formula:

$$z = \frac{a-b}{\sqrt{a+b}} \text{ where: } z > 1.96 = P < 0.05$$

Chapter III: Results and Conclusion

3.1 Single Drug Treatment

The activity of each drug, CQ, ART, DIOX, and NPC, was analyzed at varying concentrations to obtain an IC-50 value. Because the experiment was run in duplicate, the IC-50 values presented in *Table 3* below represent the mean IC-50 value obtained from the two trials.

The IC50 values recorded were as follows: For D6 CQ was 26.9 nM, ART was 70.1 nM, DIOX was 48.5 nM, and NPC was 1297.5 nM. For the W2 strain, the average IC-50 values were: CQ 355.2, ART 99.9, DIOX 77.4, and NPC 4573.8. The results are presented in *Table 3* below and recorded as a growth response curves in *Figure 6* and *Figure 7* below. The results confirm CQ-resistant property of the *Pf* W2 strain.

Table 3: IC-50 values of standard and experimental antimalarial drugs on CQ-susceptible (D6) and -resistant (W2) P. falciparum. Values are mean \pm SD of four observations

Drug	D6 IC-50 (nM)	W2 IC-50 (nM)
Chloroquine	26.9 \pm 3.8	355.2 \pm 37.4
Artemisinin	70.1 \pm 1.6	99.9 \pm 16.0
Dimer Oxime	48.5 \pm 0.1	77.4 \pm 12.4
NPC 1161B	1297.5 \pm 142.3	626.8 \pm 38.3

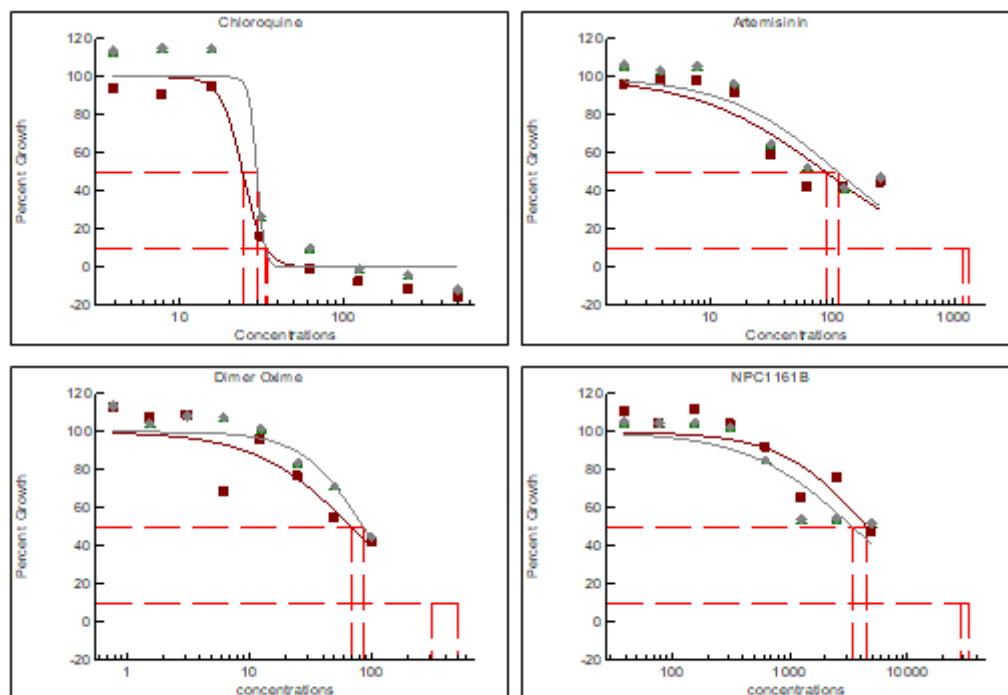


Figure 6: Growth response curves of D6 to CQ, ART, DIOX, and NPC represented in nM.

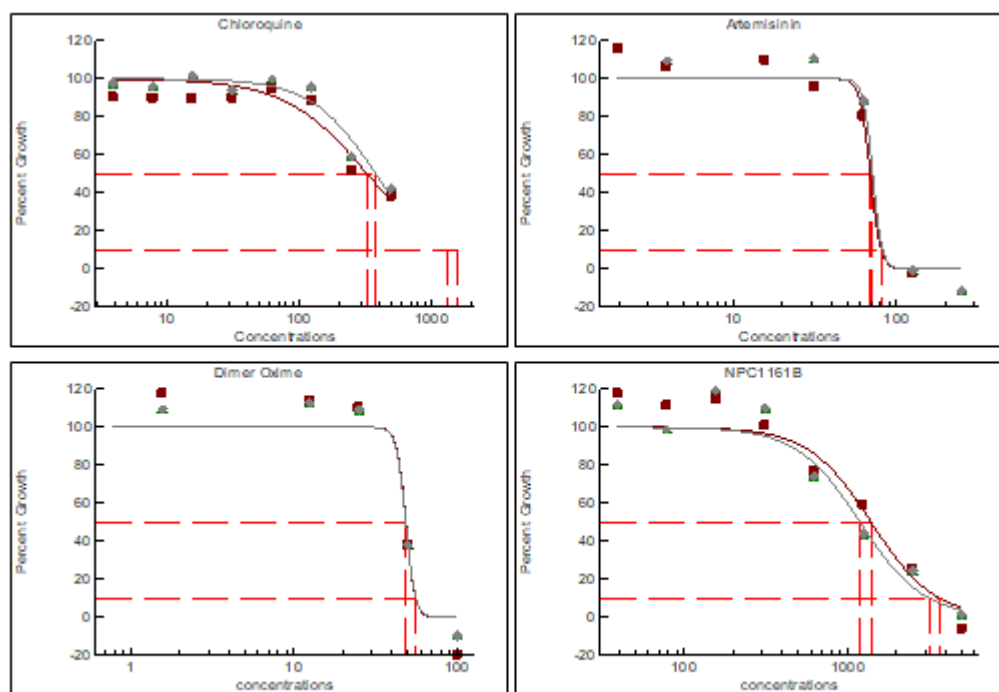


Figure 7: Growth response curve of W2 to CQ, ART, DIOX, and NPC represented in nM

3.2 Combination Assay Results

In order to assess the antimalarial activity of the drugs in combination, the SYBR Green based fluorescence assay was incorporated with a checkerboard assay of the drugs and IC-50 values calculated based on the percent growth observed from the assay.

The first combination assessed was Artemisinin with Dimer Oxime. The IC-50 recorded for the ART only column was 63.1 nM. ART was combined with DIOX over a range from 200 nM to 3.125 nM. The effects of DIOX on ART were seen starting at a DIOX concentration of 25 nM. At ART + 25 nM DIOX, the IC-50 value was 16.5 nM ($P<0.05$), ART + 12.5 nM DIOX showed an IC-50 was 41.2 ($P<0.05$), and at ART + 3.125 nM DIOX the IC-50 was 38.5 ($P<0.05$).

The isolated DIOX column showed an IC-50 value of 32.32 nM. The effects of ART on DIOX were evident starting at DIOX + 12.5 nM ART where the IC-50 was 68.544 nM ($P<0.05$), a nearly two times increase in IC-50. The results of this combination are presented in *Table 4*, and *Figure 8* below.

Table 4: The calculated IC-50 values recorded from the combination plate of the drugs Artemisinin and Dimer Oxime.

Combination	IC-50 (nM)	Combination	IC-50 (nM)
ART	63.1	DIOX	32.3
ART + DIOX 200	<3.125	DIOX+ART 200	<3.125
ART + DIOX 100	<3.125	DIOX+ART 100	<3.125
ART + DIOX 50	<3.125	DIOX+ART 50	<3.125
ART + DIOX 25	16.5 (P<0.05)	DIOX+ART 25	20.2
ART + DIOX 12.5	41.2 (P<0.05)	DIOX+ART 12.5	68.5 (P<0.05)
ART + DIOX 6.25	69.6	DIOX+ART 6.25	73.4 (P<0.05)
ART + DIOX 3.125	38.5 (P<0.05)	DIOX+ART3.125	44.3 (P<0.05)

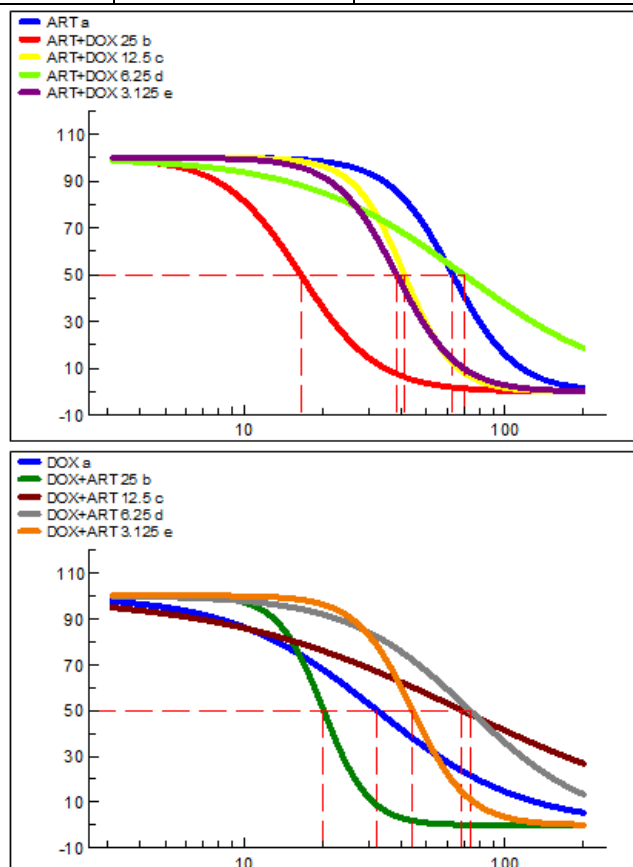


Figure 8: The growth response curves (percent growth vs. concentration) of ART in combination with DIOX (Top) and DIOX in combination with ART (Bottom).

The next combination assessed was with the drugs Artemisinin and NPC 1161B. The IC-50 recorded for the isolated ART column was 48.7 nM. ART was combined with NPC over a range from 5,000 nM to 78.125 nM. The effects of NPC on ART were seen starting at a NPC concentration of 1250 nM. At ART + 1250 nM NPC, the IC-50 value was 17.5 nM ($P<0.05$) and at ART + 312.5 nM NPC, the IC-50 was 18.9 ($P<0.05$). There was no statistically significant difference in IC-50 below this concentration.

The isolated NPC column provided an IC-50 value of 671.0 nM. The effects of ART on NPC were seen starting at NPC + 1035.2 nM ART through NPC + 3.125 nM ART. The recorded IC-50's of these four combinations were 1035.2 nM, 1352.7 nM, 1588.5 nM, and 1890.3 nM respectively (all $P<0.05$). These results also showed a nearly two-fold increase from the isolated IC-50 value of NPC. The results of this combination are presented in *Table 5*, and *Figure 9* below.

Table 5: This table represents the calculated IC-50 values recorded from the combination plate of the drugs Artemisinin and NPC 1161B.

Combination	IC-50 (nM)	Combination	IC-50 (nM)
ART	48.7	NPC	671.0
ART + NPC 5000	<3.125	NPC +ART 200	<78.125
ART + NPC 2500	<3.125	NPC +ART 100	<78.125
ART + NPC 1250	17.5 (P<0.05)	NPC +ART 50	<78.125
ART + NPC 625	18.9 (P<0.05)	NPC +ART 25	1035.2 (P<0.05)
ART + NPC 312.5	41.3	NPC +ART 12.5	1352.7 (P<0.05)
ART + NPC 156.25	48.5	NPC +ART 6.25	1588.4 (P<0.05)
ART + NPC 78.125	45.6	NPC +ART3.125	1890.3 (P<0.05)

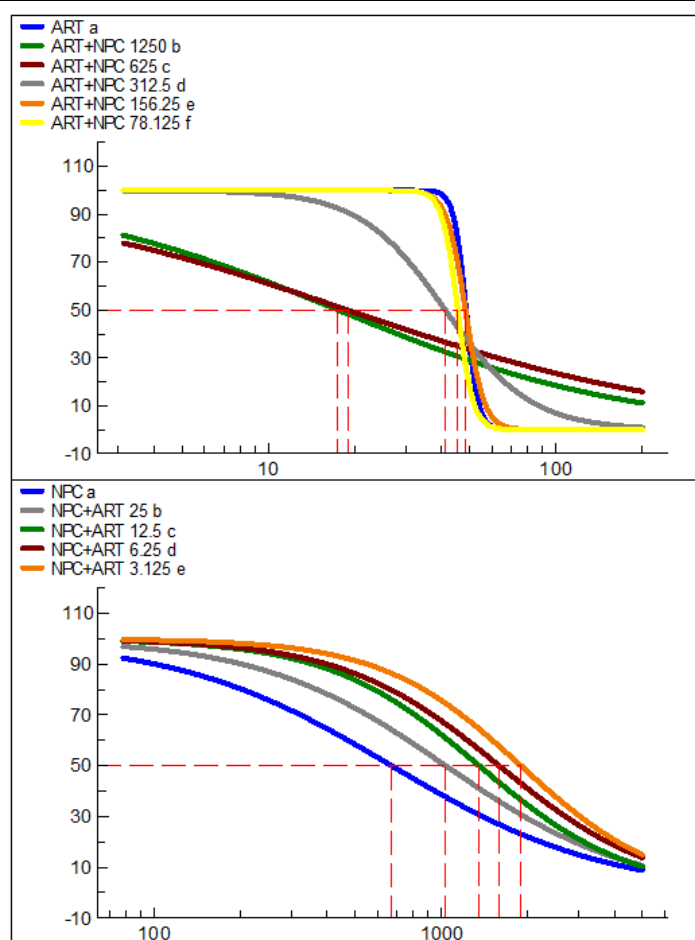


Figure 9: The growth response curves (percent growth vs. concentration) of ART in combination with NPC (Top) and NPC in combination with ART (bottom).

The next combination assessed was with the drugs Chloroquine and Dimer Oxime. The IC-50 recorded for the isolated CQ column was >200 nM because a higher concentration of CQ was needed (see discussion).

The isolated Dimer Oxime column provided an IC-50 value of 29.0 nM. The effects of CQ on DIOX were seen for every concentration combination with each IC-50 recorded nearly double that of the isolated value of DIOX (all $P < 0.05$). The results of this combination are presented in *Table 6*, and *Figure 10* below.

Table 6: This table represents the calculated IC-50 values recorded from the combination plate of the drugs Chloroquine and Dimer Oxime.

Combination	IC-50 (nM)	Combination	IC-50 (nM)
CQ	>200	DIOX	29.0
CQ+ DIOX 200	<3.125	DIOX+ CQ 200	47.7 (P<0.05)
CQ+ DIOX 100	<3.125	DIOX+ CQ 100	52.5 (P<0.05)
CQ+ DIOX 50	>200	DIOX+ CQ 50	59.9 (P<0.05)
CQ+ DIOX 25	>200	DIOX+ CQ 25	56.8 (P<0.05)
CQ+ DIOX 12.5	>200	DIOX+ CQ 12.5	55.5 (P<0.05)
CQ+ DIOX 6.25	>200	DIOX+ CQ 6.25	55.5 (P<0.05)
CQ+ DIOX 3.125	>200	DIOX+ CQ 3.125	51.4 (P<0.05)

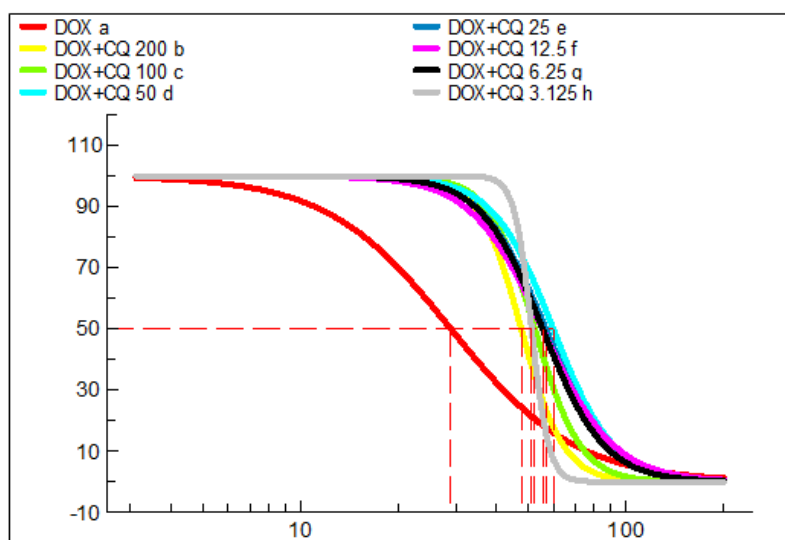


Figure 10: The growth response curves (percent growth vs. concentration) of DIOX in combination with CQ.

The final combination assessed was with the drugs Chloroquine and NPC 1161B. The IC-50 recorded for the isolated CQ column was 374.0 nM. CQ was combined with NPC over a range from 5,000 nM to 78.125 nM. The effects of NPC on CQ were seen at the combination of CQ + NPC 1250 where the IC-50 was 150.5 ($P<0.05$), and at the CQ + NPC 625 where the IC-50 was 456.3 ($P<0.05$). The remaining combinations showed no statistically significant results.

The isolated NPC column provided an IC-50 value of 607.5 nM. The effects of CQ on NPC were seen at every concentration except for NPC + CQ 200. The remaining CQ concentration combinations showed a nearly threefold increase in IC-50 value (all $P<0.05$). The results of this combination are presented in *Table 7* and *Figure 11* below.

Table 7: This table represents the calculated IC-50 values recorded from the combination plate of the drugs Chloroquine and NCP 1161B

Combination	IC-50 (nM)	Combination	IC-50 (nM)
CQ	374.0	NPC	607.5
CQ + NPC 5000	<6.25	NPC + CQ 200	<78.125
CQ + NPC 2500	<6.25	NPC + CQ 100	1616.9 (P<0.05)
CQ + NPC 1250	150.5 (P<0.05)	NPC + CQ 50	1594.5 (P<0.05)
CQ + NPC 625	456.3 (P<0.05)	NPC + CQ 25	1308.4 (P<0.05)
CQ + NPC 312.5	379.9	NPC + CQ 12.5	1653.8 (P<0.05)
CQ + NPC 156.25	380.8	NPC + CQ 6.25	1767.1 (P<0.05)
CQ + NPC 78.125	386.4	NPC + CQ 3.125	1367.1 (P<0.05)

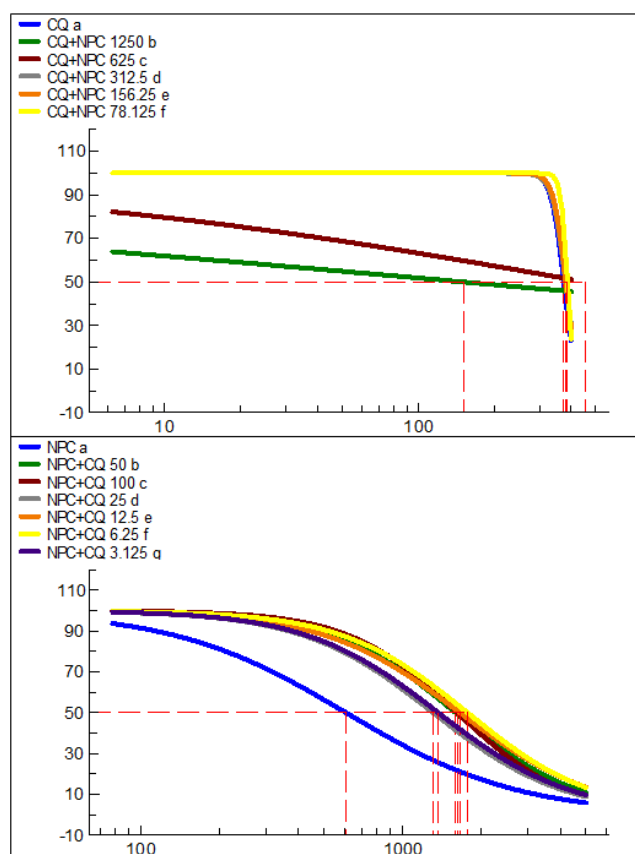


Figure 11: The growth response curves (percent growth vs. concentration) of CQ in combination with NPC (Top) and NPC in combination with CQ (bottom).

3.3 Conclusions

The results of the single treatment control assay helped to establish the drug resistant nature of the W2 strain of *P. falciparum*. The IC-50 value of CQ in the W2 strain increased from 26.9 nM in the D6 treatment to 355.2 nM in the W2 treatment. This significant increase in IC-50 value across strains confirms the CQ resistant nature of the W2 laboratory strain of *P. falciparum*.

The IC-50 of the ART + DIOX 25 nM treatment showed a statistically significant decrease in IC-50 value from 63.1 nM to 16.5 nM. This suggests that DIOX could be showing additive effects to the activity of ART *in vitro*. However, the effects of ART on the activity of DIOX suggest that partial antagonism has occurred. At DIOX + ART 25 nM and 12.5 nM, the IC-50 value is more than twice the value of the control DIOX IC-50 of 32.3 nM suggesting ART enacts partial antagonism on the activity of DIOX.

For the combination of ART and NPC, the IC-50 showed a statistically significant decrease from the control ART IC-50 value of 48.7 nM at the treatment group of ART + NPC 1250 nM and 625 nM. This decrease in IC-50 value suggests that NPC shows partial additive effects on the activity of ART. The effects of ART on NPC showed a nearly two-fold increase in IC-50 value when compared to the NPC control IC-50 value of 671.0 nM. This two fold, and in the case of NPC + ART 3.125 nM three-fold, increase in IC-50 value suggest that ART shows partial antagonism on the activity of NPC *in vitro*.

A mistake was made in the experimental setup with regard to the CQ and DIOX combination. Because of the CQ resistant nature of the W2 strain of *P. falciparum*, a higher concentration of CQ should have been used in order to obtain an IC-50 value for the experiment. However, while the concentration of CQ was low, the DIOX + CQ

combination showed a statistically significant increase in IC-50 value at all experimental concentration combinations.

For the combination of NPC + CQ, the IC-50 showed a statistically significant increase from the NPC control IC-50 of 607.5 nM for all concentrations except that of NPC + CQ 200 nM where the concentration of CQ was sufficient to kill the parasite. The two to three-fold increase in IC-50 values recorded suggests that CQ shows partial antagonism on the activity of NPC *in vitro*.

These results are the first from the lab to be reported on these drug combinations. It appears from the data that CQ and ART both antagonize the activity of NPC 1161B, however repeated studies and further evaluation need to be conducted to confirm the results. Overall the results from the single treatment test suggest that both Dimer Oxime and NPC 1161B could be effective clinical antimalarials. However, the results from the combination assays suggest that they both have the potential to show antagonism in combination with other drugs. These results should be tested again *in vitro* in order to be confirmed, and if confirmed, should then be continued to be evaluated in the mouse model to further analyze the activity.

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